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Influence of Organic Nutrients and Cocultures on the Competitive Behavior of 1,2-Dichloroethane-Degrading Bacteria

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The effects of organic nutrients and cocultures on substrate removal by and competitive behavior of 1,2-dichloroethane-degrading bacteria were investigated. *Xanthobacter autotrophicus* GJ10 needed biotin for optimal growth on 1,2-dichloroethane. In continuous culture, dilution of biotin to a concentration below 0.2 nM resulted in washout. Growth could be restored by inoculation with the 2-chloroethanol utilizer *Pseudomonas* sp. strain GJ1, leading to a new steady state in which about 1% of the mixed culture consisted of cells of strain GJ1. This indicates that strain GJ1 excreted biotin or a precursor for its synthesis. Inoculation of the mixed culture with *Ancyllobacter aquaticus* AD25 did not result in washout of strain GJ10, although strain AD25 has a 10-fold-lower K_s for growth on 1,2-dichloroethane. Strain AD25 did not become dominant because of the lack of vitamins, which are necessary for its optimal growth. The results indicate that medium composition and the presence of other species strongly influence the effect of substrate limitation on the composition of a bacterial population that degrades a xenobiotic compound in a continuous culture.

The short-chain chlorinated aliphatic compound 1,2-dichloroethane (DCE) is produced industrially in larger amounts than any other halogenated compound (13). DCE and related chemicals are volatile and often present as contaminants in industrial waste gases. If these xenobiotic compounds can be degraded, biological trickling filters may be used to decrease their release into the environment through the air (4, 5, 9). The elimination of dichloromethane from air has been achieved with such filters inoculated with dichloromethane utilizers (3–5, 9). DCE can also serve as a sole carbon and energy source for specialized methylotrophs such as *Xanthobacter autotrophicus* GJ10 (22). A trickling filter was not usable for the removal of DCE from air inoculated with this organism, however, because clogging and decreased removal efficiencies occurred within months of operation (3). The cause of this instability could be the accumulation of toxic compounds or the formation of slime and sheets by excessive biomass. Furthermore, the kinetics of substrate removal by strain GJ10 are not very attractive, which could limit the applicability (8).

A solution to such problems may be the use of DCE-utilizing organisms that have more suitable properties. A promising strain in this respect is *Ancyllobacter aquaticus* AD25, isolated on 2-chloroethylvinylether. The organism was recently shown to have a much higher affinity for DCE than strain GJ10 as a result of its higher haloalkane dehalogenase content (22). Furthermore, strain AD25 adheres quickly to glass and steel, and the production of slime was not observed (22).

The use of specific strains in continuous biological processes for waste gas treatment requires knowledge of ways to stimulate the growth of desirable organisms over those with less favorable properties. For this, insight into the factors that determine competitive behavior is needed; these may include the kinetics of substrate removal and the requirement for organic cosubstrates (7).

In this article, we report studies on the effect of organic nutrients and a coculture on the competition between *X. autotrophicus* GJ10 and *A. aquaticus* AD25. The natural population from which strain GJ10 was isolated on DCE also contained *Pseudomonas* sp. strain GJ1, which was coenriched (12). The effect of this culture on the growth of strain GJ10 and the competition between strains GJ10 and AD25 were studied. The results indicate that a mixed culture of *X. autotrophicus* GJ10 and *Pseudomonas* sp. strain GJ1 grows much more stably on DCE in continuous cultures than a pure culture of strain GJ10 and that strain GJ1 stimulates the growth of GJ10 but not of *A. aquaticus* AD25.

MATERIALS AND METHODS

Organisms and growth conditions. *X. autotrophicus* GJ10 (11) and GJ10MR1 (23) and *A. aquaticus* AD25 (22) were maintained on nutrient broth (NB) or brain heart infusion (BHI) agar plates. *Pseudomonas* sp. strain GJ1 (10) was maintained on NB agar plates.

The mineral medium (MM) used for continuous cultures contained (per liter) 0.16 g of KH_2PO_4 , 0.46 g of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 0.2 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g of $(\text{NH}_4)_2\text{SO}_4$, and 5 ml of trace metals solution (12, 22). The phosphate buffer was autoclaved after it was brought to pH 5.0 with 3 M H_2SO_4 . The other components were autoclaved separately.

For batch cultures and solid media, the same MM was used but with a fivefold-higher concentration of the phosphates (22). Yeast extract and a mixed vitamin solution (12) were added where indicated. Flasks were filled to one-fifth of their volume with medium and closed with viton rubber septa. Cultures were incubated at 30°C with rotary shaking (22).

Growth rates were determined by measuring the increase in the A_{450} of cultures growing in MM supplemented with yeast extract (30 mg/liter). For strain AD25, which grew in flocks, the A_{450} was determined after destruction of the flocks by pulling 8 ml of culture fluid through a syringe (volume, 10 ml) 20 times with a needle that had a diameter of

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1 mm. No significant growth occurred on MM containing yeast extract (30 mg/liter) in the absence of an additional carbon source ($A_{450} < 0.05$).

Chloride levels in culture media were measured by the method used before (22).

Continuous cultures. Continuous cultivation of bacteria was done in a fermentor with a volume of 1 liter, which was filled with 750 ml of medium as described previously (23). Culture medium was supplied via silicon rubber tubes and a Cole-Parmer peristaltic pump, model 7554-30. The reactor was stirred with a double-blade rotor at 800 rpm unless stated otherwise. The chemostat cultures were maintained at pH 7 by automatic addition of sterile 2 N KOH. The reactor was kept at 30°C with a temperature sensor and controller connected to an infrared lamp.

DCE was supplied via the gas phase as follows (23). To minimize evaporation of medium from the culture vessel, air was bubbled through a water column that had a length of approximately 10 cm. A second air flow was bubbled through a flask containing halogenated substrate. Both gas flows were adjusted by independent flow controllers. The total gas flow rate was 900 to 3,500 ml/h, depending on the desired biomass concentration. The substrate air flow rate was 60 to 500 ml/h, and the molar ratio of oxygen to DCE which entered the fermentor was always at least 20:1.

Samples taken from the continuous culture had a volume of approximately 8 to 12 ml and were collected in sterile glass tubes containing 0.5 ml of concentrated phosphoric acid to rapidly inactivate the cells. Maximum growth rates on DCE in the fermentor were measured under fed-batch conditions by continuous addition of DCE via the gas phase as described previously (23).

To investigate the composition of mixed cultures, the number of cells was estimated microscopically with a counting chamber and by plating diluted culture samples on NB and on BHI agar. Because of its characteristic circular cell shape (22), strain AD25 could be distinguished from strains GJ10 and GJ1 by microscopy. Strain GJ1 could be distinguished from strain GJ10 because it formed nonpigmented colonies after 2 days of growth on NB agar. Strain GJ10 formed yellow colonies which became visible after 4 days. Strain AD25 grew only on BHI agar and produced white colonies after 1 week of incubation at 30°C.

Growth kinetics. The Monod half-saturation constant was determined by analysis of the substrate concentrations in the water phase of cultures growing at a dilution rate of $0.5 \mu_{\max}$. Data were collected after at least five volume changes had occurred after adjustment of the dilution rate (23).

Gas chromatography. Halogenated compounds were quantitatively determined by capillary gas chromatographic analysis. The equipment and temperature program were as described before (23). Culture samples (4.5 ml) were extracted with 1.5 ml of diethylether containing 0.05 mM 1-bromohexane as the internal standard. The upper layer was analyzed by split injection of 1- μ l samples into the gas chromatograph.

The DCE concentrations in the ingoing and outgoing gases of the continuous culture were analyzed by injection of gas samples (200 μ l) into the gas chromatograph. Calibration of gaseous samples of DCE was performed as described previously (23). The reported gas phase concentrations are the averages of at least four separate analyses. The maximum variation in concentration between these samples was less than 10%.

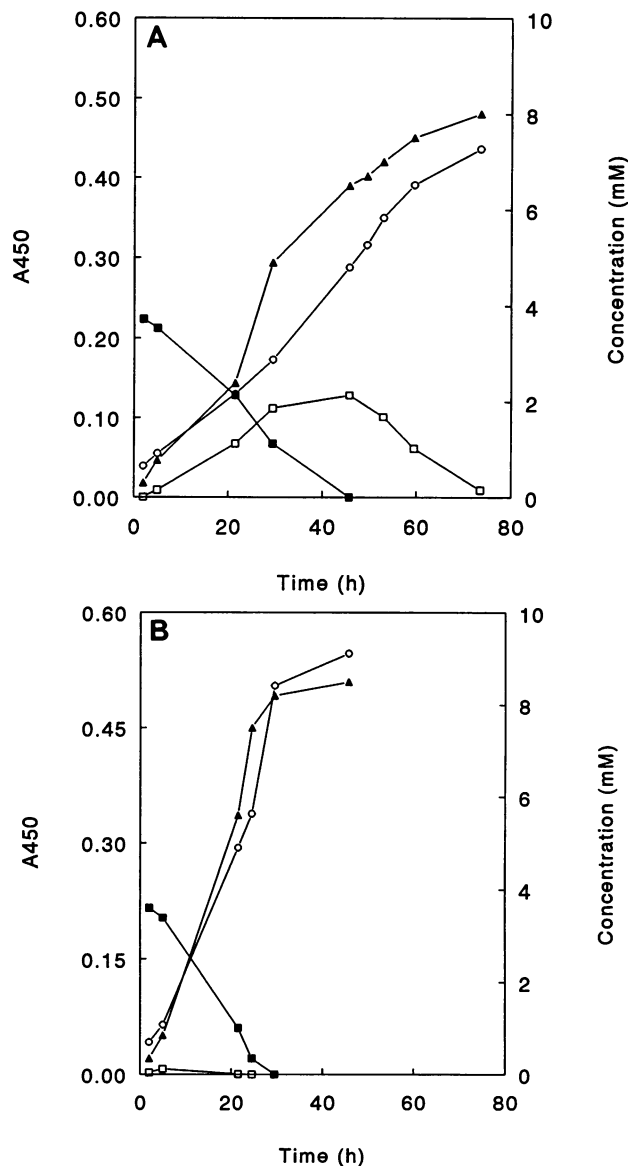


FIG. 1. Growth parameters of strain GJ10 on 4 mM DCE in batch cultures supplemented with yeast extract (10 mg/liter) (A) or vitamins (B). Symbols: ○, A_{450} ; ■, DCE; □, 2-chloroethanol; ▲, chloride.

Chemicals. The chemicals used in this study were all obtained from commercial sources (Janssen Chimica and Aldrich) and were checked for purity as stated before (22).

RESULTS

Effect of organic nutrients on DCE utilization. The effect of vitamins and yeast extract on the growth of *X. autotrophicus* GJ10 with DCE was examined with batch cultures. In the presence of yeast extract (10 mg/liter), strain GJ10 grew initially with a μ_{\max} of $0.04 \pm 0.01 \text{ h}^{-1}$, followed by nonexponential growth (Fig. 1). During DCE degradation, 2-chloroethanol accumulated up to 2.2 mM and was slowly utilized. The addition of vitamins resulted in a μ_{\max} of $0.09 \pm 0.01 \text{ h}^{-1}$ and reduced the accumulation of 2-chloroethanol

TABLE 1. Growth parameters of *X. autotrophicus* GJ10 on DCE

Addition to culture	Dilution rate (h ⁻¹)	Chloride concn (mM)	DCE _{water} (μM) ^a	DCE _{air} (μmol/h) ^b		μ _{max} (h ⁻¹)	K _s (μM)	Yield ^c (mg/mg)
				In	Out			
Yeast extract (30 mg/liter)	0.052	5.7	279	139	24.5	0.104	260	0.24
Biotin (12 μg/liter)	0.055	15.0	283	350	34	0.105	260	0.22
Strain GJ1	0.036	34	130	400	25	ND ^d	ND	0.21

^a DCE concentration in the water phase.^b DCE flows via the air phase.^c Growth yield, in milligrams of cells (dry weight) per milligram of DCE.^d ND, not determined.

(Fig. 1). In the presence of biotin, strain GJ10 grew with a μ_{max} of 0.105 ± 0.02 h⁻¹. Thus, strain GJ10 required biotin for optimal growth.

An even stronger effect of vitamins was detected for strain GJ10MR1, a mutant described previously (23). This strain was not able to grow on methanol or DCE or to liberate chloride from DCE in the absence of vitamins or nutrients. Replica plating on plates with citrate (5 mM) as the carbon source showed that growth could be restored by adding biotin. Thus, biotin stimulated the growth both of strain GJ10 and of strain GJ10MR1, with the difference that strain GJ10MR1 had an absolute requirement for this vitamin. Strains GJ10 and GJ10MR1 were also found to be capable of growth on DCE with a mixed culture of soil organisms or with the 2-chloroethanol utilizer *Pseudomonas* sp. strain GJ1 added, suggesting that growth could be stimulated through cross-feeding by other organisms.

Growth of strain GJ10 on DCE in continuous cultures. The effect of nutrients on the growth of strain GJ10 was investigated further with fermentor cultures. The μ_{max} with DCE was 0.104 h⁻¹, as determined under fed-batch conditions in the presence of 30 mg of yeast extract per liter (23). Both biotin (12 μg/liter) and yeast extract (30 mg/liter) could be used to obtain stable growth of strain GJ10 in continuous culture. The Monod half-saturation constants in the presence of biotin and of yeast extract were almost identical (Table 1).

In the absence of organic nutrients, strain GJ10 grew with a μ_{max} of less than 0.03 h⁻¹ under fed-batch conditions. After the medium pump was set at a *D* of 0.050 h⁻¹, the culture washed out, as expected, leading to a decrease in cell density from 0.1 to 0.01 mg (dry weight) per ml after 70 h. The cells that remained in the culture vessel no longer grew, even when the system was switched back to fed-batch conditions. The concentrations of DCE and 2-chloroethanol increased to 3.8 and 1.2 mM, respectively, which is still below the level of toxicity. After 3 days of batch cultivation, a viable-cell count showed that 9.1 × 10⁶ cells per ml produced colonies on NB agar plates. This drop in activity was observed repeatedly with strain GJ10, even in medium containing yeast extract (30 mg/liter) under stress conditions, such as a pH drop (pH 3), and after overnight incubation at 40°C. Addition of biotin, yeast extract, or a new culture of cells to such cultures did not restore growth on 2-chloroethanol or DCE. The results indicate a requirement for organic growth factors for stable growth and the formation of a growth-inhibiting component(s) by strain GJ10 from DCE under unfavorable conditions.

Effect of *Pseudomonas* sp. strain GJ1 on growth of strain GJ10. The growth of strain GJ10 on DCE in a continuous culture with 12 μg of biotin per liter added to the influent

medium was monitored for a longer period at a dilution rate of 0.030 h⁻¹ (Fig. 2). During the first 15 days, the culture was not in a steady state because of problems with the DCE and air flows but 2-chloroethanol was not formed. After this period, the culture reached a stable steady state, and the DCE concentration in the water phase became 80 ± 10 μM

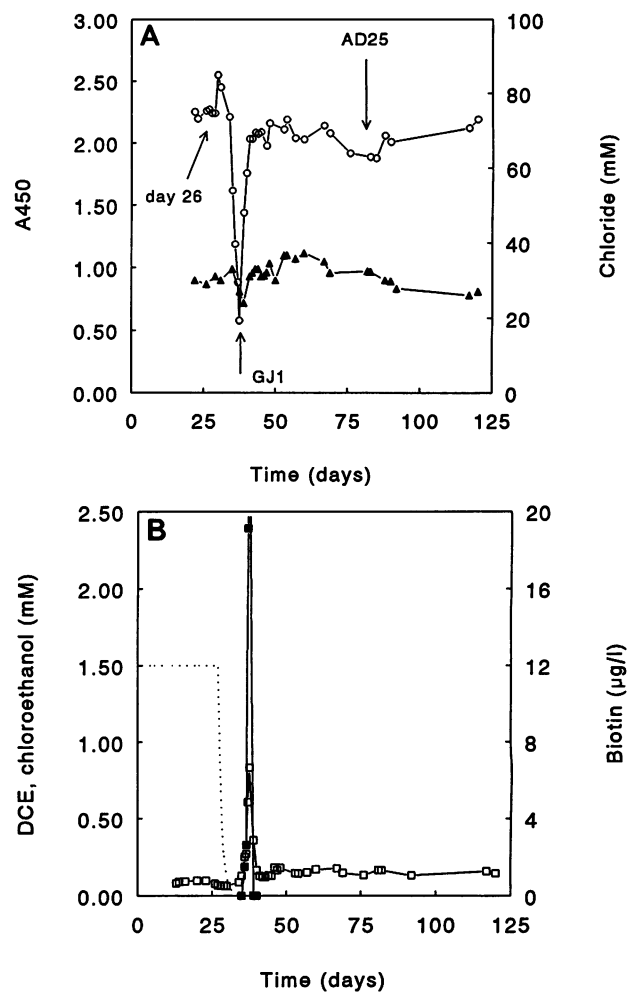


FIG. 2. Growth parameters of strain GJ10 in continuous culture on DCE. The biotin supply was stopped on day 26, strain GJ1 was added on day 37, and strain AD25 was added on day 80. Symbols: (A) ○, A₄₅₀; ▲, chloride; (B) □, DCE in the water phase; ■, 2-chloroethanol; ···, calculated biotin concentration.

(Fig. 2). The effect of biotin limitation was studied by replacing the medium supply vessel on day 26 with a vessel containing medium without biotin. On day 34, the culture started to wash out, and slight foaming occurred. The biotin concentration (C_t) at this timepoint (t) was calculated to have dropped from 12 to 0.046 $\mu\text{g/liter}$, assuming an exponential decrease by washout according to the formula $C_t = C_0 \times e^{-Dt}$, where D is the dilution rate. The decrease in biomass levels was accompanied by a sharp increase in the concentrations of DCE and 2-chloroethanol in the water phase, which reached levels of 0.83 and 3.0 mM, respectively, on day 37. The washout rate observed over days 34 to 36 was -0.013 h^{-1} , which means that the culture still grew with a μ of 0.017 h^{-1} .

On day 37, the A_{450} had dropped to 0.6, and 1 ml of a culture of *Pseudomonas* sp. strain GJ1 (0.09 mg of cells [dry weight] per ml) was inoculated into the fermentor (Fig. 2). The culture of strain GJ1 was first grown in a batch on 5 mM 2-chloroethanol in the absence of yeast extract. Within 2 days, the 2-chloroethanol in the continuous culture had been completely utilized, and an increase in the cell density became visible. On day 45, a new steady state was reached, with a DCE concentration in the water phase of $130 \pm 20 \mu\text{M}$, which is somewhat higher than observed during the former steady state. This may be due to a slightly higher dilution rate (0.036 h^{-1}).

The mixed culture of strains GJ10 and GJ1 grew stably for weeks on DCE in continuous cultures in the absence of yeast extract, biotin, and vitamins. The total number of cells, determined by microscopic counting with a counting chamber, was $1.8 \times 10^9/\text{ml}$. Plating experiments on NB agar showed that the culture was composed of 10^9 and 10^7 CFU of strain GJ10 and strain GJ1, respectively, per ml, indicating that about 1% of the cells of the culture were of strain GJ1.

Inoculation of strain AD25. Previously, it was shown that because of the much better affinity for DCE, *A. aquaticus* AD25 was able to outcompete strain GJ10 during growth on DCE in a continuous culture in the presence of 30 mg of yeast extract per liter (22). To investigate whether strain AD25 would outcompete strain GJ10 with strain GJ1 present in medium without yeast extract or vitamins, a 200-ml culture of strain AD25 grown on 2.5 mM DCE was added to the fermentor on day 80 (Fig. 2). During the next weeks, the concentration of DCE in the water phase did not change significantly (up to day 120). The numbers of cells of strains GJ10 and GJ1 remained at $10^9/\text{ml}$ and $10^7/\text{ml}$, respectively. Viable-cell counts of strain AD25 revealed cell numbers of $2 \times 10^7/\text{ml}$ after 14 days, which was confirmed by microscopic counts.

After the inoculation of strain AD25, the mixed culture had the tendency to flocculate. At day 100, yellow-colored wall growth became visible. The concentration of DCE in the water phase at day 120 was still about $130 \mu\text{M}$. Strain AD25 thus did not become the dominant species within 5 weeks.

Growth of strain AD25 in batch cultures. The μ_{max} of strain AD25 growing on DCE in continuous cultures with 30 mg of yeast extract per liter is 0.098 h^{-1} (23). Under these conditions, the K_s of the organism is $24 \mu\text{M}$, which is about 10 times lower than observed for strain GJ10 (23). To investigate why strain AD25 did not become dominant in the mixed culture, the effects of organic nutrients and strain GJ1 on the growth of strain AD25 were studied.

Strain AD25 grew in batch cultures on DCE in the absence of yeast extract with a μ_{max} that was twofold lower than that with yeast extract present (Table 2). In contrast to strain

TABLE 2. Growth of strain AD25 in batch cultures on 4 mM DCE

Addition to culture	μ_{max} (h^{-1})	2-Chloroethanol accumulated (mM)	
		30 h	75 h
None	0.02	3.2	3.0
Strain GJ1	0.02	1.9	0.0
Biotin (12 $\mu\text{g/liter}$)	0.02	3.5	3.0
Vitamins (1 ml/liter)	0.04	2.0	0.0
Yeast extract (30 mg/liter)	0.04	2.5	0.0

GJ10, addition of biotin to the medium did not increase the growth rate of strain AD25. As with strain GJ10, poor growth of strain AD25 was accompanied by accumulation of high levels of 2-chloroethanol (Table 2), which was utilized during further growth.

Strain GJ1 did not stimulate the growth of strain AD25 on DCE. The amount of 2-chloroethanol which accumulated was low, probably because of utilization by strain GJ1. The growth of strain AD25 was stimulated by the addition of vitamins to the medium, but biotin alone did not have an effect. Apparently, strain GJ1 was not able to overcome the requirements for organic nutrients, as it did with strain GJ10.

Mutants of strain GJ10. During growth of strain GJ10 on DCE in continuous cultures, the development of mutants which showed a different type of colony morphology on NB agar plates was repeatedly observed. The cells formed rough dry colonies instead of smooth slimy colonies and had a darker yellow color. One of these mutants, designated strain GJ10MS, produced tiny colonies which became visible after 10 days of incubation and produced less slime on NB agar plates. The mutants eventually became dominant after 30 to 40 days. There were no differences in the growth rates of strains GJ10MS and GJ10 in MM supplemented with yeast extract (30 mg/liter) and 4 mM DCE or 2-chloroethanol. After two subcultivations in liquid medium followed by streaking on NB agar plates, revertant colonies were observed. Replica plating experiments showed that strain GJ10MS did not possess other auxotrophic traits than the wild-type strain.

DISCUSSION

There is considerable interest in the use of specific xenobiotic-degrading bacterial cultures in bioreactors for environmental cleanup purposes. Such applications require that the introduced organisms be maintained long enough to display their biodegradative capacities. For continuous processes, this will require competition with endogenous organisms for growth-limiting substrates. Little is known about the microbial factors that determine the effect of influent characteristics and process conditions on the composition of microbial populations, with the exception of the importance of overall physiological characteristics, such as electron acceptor usage and the role of kinetics (6, 7). Using chemostat cultures, in which competitive effects can be simulated, we have investigated the effect of organic nutrients and a chloroethanol-utilizing *Pseudomonas* sp. on the stability and competition of DCE-degrading bacteria.

We have carried out these studies with different strains of *X. autotrophicus* and *A. aquaticus* that possess the same route for DCE degradation (11, 12, 22). Haloalkane dehalogenase, which catalyzes the first catabolic step, hydrolyti-

cally converts DCE to 2-chloroethanol, which can be used as a growth substrate by several pure cultures (12, 19, 20). The dehalogenase is identical in different strains (22), and its expression level influences the K_s of DCE degraders (23). Previous kinetic studies with growing cultures indicated that the bacterial cell envelope did not act as a barrier for the permeation of DCE (22). The 2-chloroethanol that is produced is exported to the periplasmic space, where it is oxidized by a periplasmic quinoprotein, alcohol dehydrogenase (10). In the absence of nutrients or yeast extract, both strains GJ10 and AD25 grew more slowly and accumulated 2-chloroethanol in the medium during growth on DCE in batch cultures. Apparently, under these conditions the rate of alcohol oxidation is lower than when vitamins are present and the cells do not shut off 2-chloroethanol formation. The conversion of DCE under suboptimal conditions thus was limited at the level of 2-chloroethanol oxidation, which may help to prevent the formation of chloroacetaldehyde, which is toxic for DCE-utilizing organisms (22). The accumulation of chloroacetaldehyde in cultures of strain AD25 seems unlikely because of the high chloroacetaldehyde dehydrogenase activity, 1.87 U/mg of protein, which is more than 10 times higher than that found for strain GJ10 (22).

During prolonged cultivation of strain GJ10 in the absence of biotin in continuous cultures, toxic compounds accumulated from DCE. Inoculation with *Pseudomonas* sp. strain GJ1 in a subsequent experiment resulted in the restoration of growth on DCE and eventually in a new steady state. Thus, a mixed culture of strains GJ10 and GJ1 grew more stably on DCE than did strain GJ10 alone. Strain GJ1 fulfilled the requirement of strain GJ10 for biotin, which has also been observed by others for *Xanthobacter* strains (24). In addition, strain GJ1 degraded the toxic compounds that accumulated under suboptimal conditions and inhibited further growth of strain GJ10.

Examples of mutualistic interactions between microorganisms that degrade xenobiotics have been observed before (18). A mixed culture consisting of a *Nocardia* sp. and a *Pseudomonas* sp. grew on cyclohexane (18). Neither of the pure cultures could grow on this compound, although the *Nocardia* strain was able to oxidize cyclohexane. Growth of this organism was possible when biotin or an autoclaved supernatant of the mixed culture was added. It was concluded that biotin, and probably also other growth factors, was excreted by the *Pseudomonas* sp., which in turn grew on cell lysis products or cyclohexane catabolic products. A mixed culture of two *Streptomyces* spp. and an unidentified bacterial culture, designated strain 3CI, was capable of growth on trichloroacetic acid (13). Neither of the three strains could grow alone on trichloroacetic acid, but strain 3CI could dehalogenate trichloroacetic acid. Growth occurred when the organism was provided with vitamin B₁₂ by one of the *Streptomyces* species.

During growth of strain GJ10 on DCE in continuous cultures, non-slime-producing mutants developed and ultimately became dominant. The formation of this kind of mutant was not reported by others (8). Slime production has been recognized as a general characteristic of *Xanthobacter* strains (20, 23), and a mutant that produces less slime has been isolated (1). The loss of slime production may result in more efficient growth on DCE in continuous culture, allowing the mutants to become dominant (7). The occurrence of spontaneous mutants under selective chemostat conditions is not uncommon (6).

In previous continuous-culture experiments, *A. aquaticus* AD25 outcompeted strain GJ10 during growth on DCE in the

presence of 30 mg of yeast extract per liter (23). This was possible because strain AD25 has a K_s of 24 μ M and strain GJ10 has a K_s of 260 μ M (23). However, strain AD25 needed vitamins or yeast extract for optimal growth, and neither biotin nor strain GJ1 stimulated the growth of this organism, which explains why strain AD25 was not able to compete successfully for DCE in the mixed culture of strains GJ10 and GJ1. These results indicate that, in addition to kinetic factors, the presence of other species and organic growth factors may strongly influence the type of organism that becomes dominant in a treatment system and thereby the removal efficiency and overall stability of the system.

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